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14. ABSTRACT

The CD4+ T cell response is critical for cellular autoimmunity in human T1D, but incomplete understanding of issues of specific cell frequency, avidity, function, and correlation with disease status presents major obstacles to improved therapies. This research study entails using humanized mice manifesting type 1 diabetes (T1D)-associated human HLA molecules to address the fate and pathogenicity of high and low avidity T cells reactive to the putative autoantigen glutamic acid decarboxylase 65 (GAD65). By modeling the dominant human anti-GAD65 response in HLA- and TCR-transgenic mice, we proposed to determine whether pathogenic and/or regulatory responses correspond to high or low avidity profiles at different points during disease course. These ongoing studies indicate that the tolerance mechanisms used to prevent self-antigen GAD65 reactive T cells from eliciting autoimmunity in humanized DR4 HLA mice are diverse and that no single mechanism is exclusively used to maintain immune tolerance and prevent diabetes.

15. SUBJECT TERMS
Autoimmunity; type 1 diabetes; humanized mouse model; T cell; GAD65

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Humanized in vivo Model for Autoimmune Diabetes
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Benaroya Research Institute
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January 2008

Research Technical Report—Year 1

INTRODUCTION

The CD4+ T cell response is critical for cellular autoimmunity in human T1D, but incomplete understanding of issues of specific cell frequency, avidity, function, and correlation with disease status present major obstacles to improved therapies. This research study entails the use of humanized mice demonstrating type 1 diabetes (T1D)-associated human HLA molecules to address the fate and pathogenicity of high and low avidity T cells reactive to the putative autoantigen glutamic acid decarboxylase 65 (GAD65). By modeling the dominant human anti-GAD65 response in HLA- and TCR-transgenic mice, we proposed to determine whether pathogenic and/or regulatory responses correspond to high or low avidity profiles at different points during disease course.

BODY

Much of the focus in the first year of this grant has been on addressing Aim I: "To test the hypothesis that high avidity autoreactive $CD4^+$ T cells escape from selection and persist in the periphery as dominant clonotypes; to evaluate the fate and pathogenecity of high and low avidity autoreactive T cells representative of the human T1D repertoire."

Our models of high and low avidity autoreactive GAD65 $CD4^+$ T cells in diabetes-associated HLA transgenic mice are represented by two human TcR transgenic mouse lines we have developed. For this project, we generated mice transgenic for human TcR, which corresponds to either high avidity (TcR164, Vα12,Vβ5.1) or low avidity (TcR200, Vα4, Vβ5.1) receptors used in human subjects for CD4+ T cell responses to GAD. Both of these TcR came from clones derived from the same subject, who was HLA-DRB1*0401, so we crossed these TcR transgenic mice onto the DRB1*0401 strain for our partially humanized model.

Several potential mechanisms control self-antigen-reactive T cells that have the potential to mediate peripheral tissue damage. These include: (1) deletion, (2) modulation of the CD4/CD8 selection pathway, (3) anergy, (4) down-modulation of the T cell receptor, and (5) selection into regulatory cells, including: (a) $FoxP3^+$ Treg and (b) IL-10-producing Tr1 cells. In the first year of our studies, we found that tolerance in 164 TcR DR4 mice to the high avidity GAD65 self-antigen-reactive TcR is mitigated through several of these mechanisms. 164 thymocytes undergo strong negative selection in the thymus by deletion, resulting in low thymic cellularity (Fig. 1a., Gebe et al., manuscript attached), down-modulating CD4 and skewing these T cells into the CD8 pathway (Fig. 1d, Gebe et al., manuscript attached, also seen in 164 mice on a $Rag2^{o/o}$ background), and also by down-modulating their T cell receptor (Fig. 9). Neither anergy nor differentiation into Treg or Tr1 cells appears to be an active mechanism of tolerance for these high avidity self-antigen-reactive thymocytes, as peripheral T cells are reactive to antigenic stimulation (Fig. 3a, Gebe et al., manuscript attached), and the vast majority of Treg cells (FoxP3+) are not clonotypic (Fig. 2b, Gebe et al., manuscript attached).

In contrast, 4.13 TcR transgenic mice select T cells to near normal levels in the thymus, and peripheral cellularity is near to that in non-TcR transgenic mice (Fig. 8a). Skewing of thymocytes to the CD8 pathway, down-modulation of the TcR (Fig. 9), and deletion are not apparent in the mechanisms used in maintaining tolerance to this TcR. In spite of these differences, both 164 and 4.13 T cells respond to antigenic stimulation (Fig. 10).

In a cytokine analysis of high and low avidity, TcR T cells to GAD65 555-567 we observed that 164 T cells are of a T_H1 type secreting IFN-γ and no IL-17, IL-10, IL-4, IL-5, and little IL-2 or TNF-α (Fig. 3b, Gebe et al., manuscript attached), while 4.13 TcR T cells in contrast secrete IFN-γ and IL-10, no IL-2, IL-5, and little IL-4 and TNF-α (Fig. 8b). Preliminary data from intracellular staining suggest that IFN-γ and IL-10 are made from distinct T cells, and thus peripheral 4.13 TcR T cells display either Tr1 or T_H1 phenotypes. These data indicate that two autoreactive TcRs, both using Vα12.1/Vβ5.1 and reactive to the same naturally processed GAD65 epitope, are tolerized in DR4 mice with very distinct mechanisms.

Interestingly, although low avidity 4.13 T cells were selected well and populated the peripheral organs to near normal levels, we have not observed any indication of an islet T cell infiltrate. Studies in the upcoming year will focus on this issue, which has therapeutic implications. Preliminary data for this grant indicated that the high avidity 164 TcR transgenic mice on a Rag2-/- background were capable of mediating a mononuclear infiltrate into the islet. We now know that: (1) the cellular infiltrate, which is predominantly seen in female mice, is comprised of the 164 $CD4^+$ TcR T cells (Fig. 6a, Gebe et al., manuscript attached); and (2) the islet infiltrate is correlated with a loss in detectable beta cell insulin and an impaired response to glucose in an intra-peritoneal tolerance test (IPGTT) (Fig. 6b, Gebe et al., manuscript attached).

This is the first time that T cell reactivity to the autoantigen GAD65 has been shown to mediate a loss in islet function in a humanized mouse model. High avidity GAD65 555-567-reactive T cells can migrate to the pancreatic islet and mediate a loss in pancreatic beta cell function (impaired tolerance to exogenous glucose) and reduce beta cell insulin in infiltrated islets. The functional difference in glucose tolerance between the 164 transgenic mouse and the 4.13 transgenic mouse may be explained partly by the finding that 164 TcR cells exhibit a T_H1 cytokine profile while the 4.13 cells display a combination of both T_H1 and Tr1 cells, with the latter producing the immunosuppressive cytokine IL-10 upon stimulation.

KEY RESEARCH ACCOMPLISHMENTS

- High avidity GAD65 555-567-reactive T cells can escape from a strongly negatively selecting environment in the thymus and populate the periphery in humanized DR4 mice (DR4/164 mice).

- The high avidity peripheral GAD65 555-567-reactive T cells in DR4 mice display a $CD44^{HI}/CD62L^{Low}$ activated phenotype and a proinflammatory T_H1 profile.

- The mechanism of tolerance to high avidity GAD65-reactive T cells in DR4/164 mice is predominantly through deletion, skewing to a CD8+ phenotype and down-modulation of the TcR, not selection into a regulatory pathway.

- High avidity GAD65 555-567-reactive T cells can mediate a loss in pancreatic beta cell function and is strong evidence that GAD65 is an autoantigen capable of mediating beta cell damage in humanized DR4 mice.

- Unmanipulated high avidity GAD65 555-567-reactive T cells in humanized DR4 mice are proinflammatory and not regulatory.

- Low avidity 4.13 T cells are positively selected in humanized DR4 mice, and tolerance to the self-antigen GAD65 may be mediated by the differentiation of a portion of these T cells into a Tr1 (IL-10-secreting) regulatory pathway.

REPORTABLE OUTCOMES

Oral Presentation

Two Humanized HLA-DR4 GAD65 TCR Transgenic Mouse Lines with Similar T-cell Receptors Model Different Autoimmune Tolerance Mechanisms, 2007 FASEB Summer Conference on Autoimmunity, July 14-19, 2007, Vermont Academy, Saxtons River, VT.

Manuscripts

1. Gebe JA, Unrath KA, Yue BB, Miyake T, Falk BA, Nepom GT. 2007. **Autoreactive human T-cell receptor initiates insulitis and impaired glucose tolerance in HLA DR4 transgenic mice**, J Autoimmun (2007), doi:10.1016/j.jaut.2007.08.001 *in press.*

2. Gebe JA, Falk B, Unrath K, Nepom GT. 2007. **Autoreactive T cells in a partially humanized murine model of T1D**. Ann NY Acad Sci 1103:69-76.

CONCLUSION

Our ongoing studies indicate that the tolerance mechanisms used to prevent self-antigen GAD65-reactive T cells from eliciting autoimmunity in humanized DR4 HLA mice are diverse and no single mechanism is exclusively used to maintain immune tolerance and prevent diabetes. The avidity of the TcR-peptide-MHC complex appears to be a key determinant of which mechanisms are used. For high avidity TcR-peptide-MHC interactions, we find that the mechanisms of thymic deletion, down-modulation of the TcR, and skewing of CD4 cells to a CD8 pathway are active to minimize the peripheral occupation of these cells. In contrast, a TcR with identical Vα/Vβ usage, but of lower TcR-peptide-MHC avidity, uses entirely different mechanisms. Low avidity 4.13 GAD65-reactive T cells undergo what appears to be normal passage through the selecting thymus, but a fraction are differentiated into Tr1 cells. Data thus far indicate that the few high avidity 164 T cells that escape the strong thymic tolerance are capable of mediating pancreatic islet beta cell damage. Whether low avidity 4.13 T cells are capable of mediating beta cell damage remains an unanswered question that we hope to answer in the near future, now that these mice are bred onto a C57Bl/6 background.

It is apparent that both normal and autoimmune individuals harbor autoreactive T cells within their immune repertoires that are potentially capable of mediating an autoimmune response resulting in tissue damage. Regardless of what tolerance mechanism has failed in diabetes patients, CD4 effector T cells are involved, and targeted intervention specific to these T cells requires knowledge of their properties. Our initial results support Aim 1 in that high avidity T cells to GAD65 can escape thymic

tolerance and exist in the peripheral pool and are also capable of mediating beta cell damage

Understanding the T cell response profile in T1D will improve opportunities for advances in three major areas: (1) Monitoring pre-diabetic at-risk subjects for immune activation and signs of progression to disease; (2) Developing biomarker technology for monitoring new therapeutics targeted at the appropriate T cell specificities and phenotypes; and (3) basic knowledge regarding the nature of autoimmune T cells, most specifically the relationship between avidity and autoreactivity.

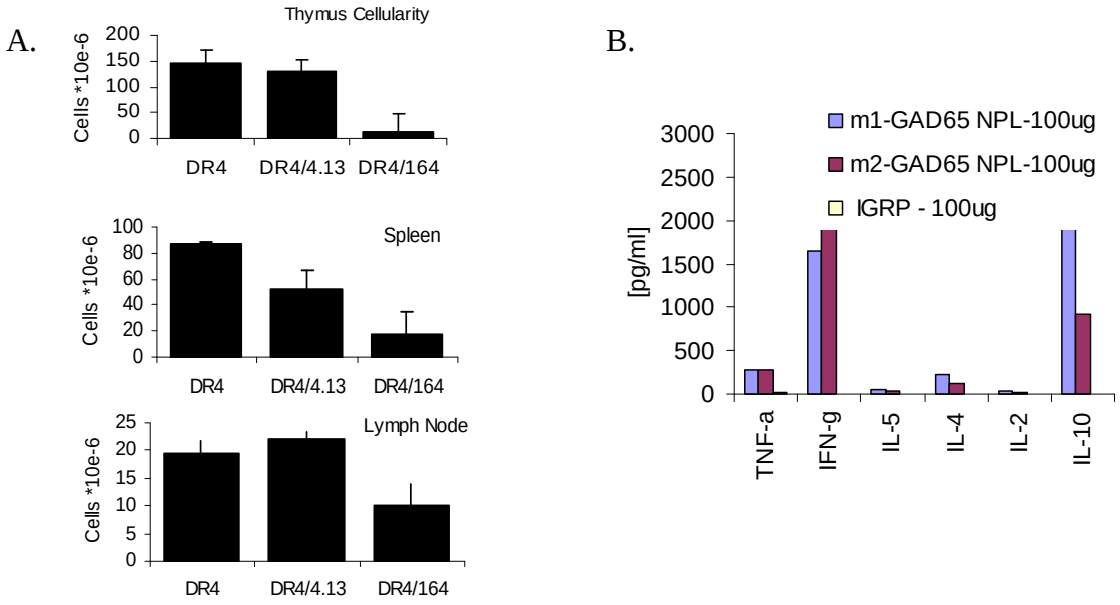

Figure 8. Tissue cellularity in DR4, DR4/164, and DR4/4.13 mice. (A) Cytokine secreted by lymph node cells from two DR4/4.13 mice to GAD65 555-567 stimulation. Cytokine profile in DR4/4.13 mice stimulated with GAD65 peptide. (B) Cytokines were assayed at 72 hours using a BD CBA T_H1/T_H2 kit.

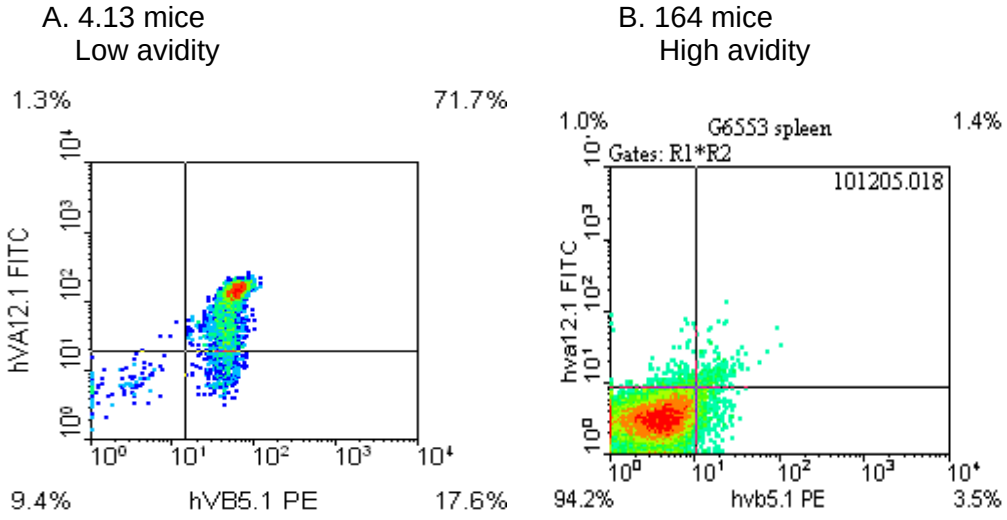

Figure 9. Vα12.1 and Vβ5.1. TcR expression in low (A) and high (B) avidity $CD4^+$ gated GAD65-responsive T cells. Spleen cells were derived from $Rag2^{o/o}$ mice to ensure all T cells express only the transgenic TcR.

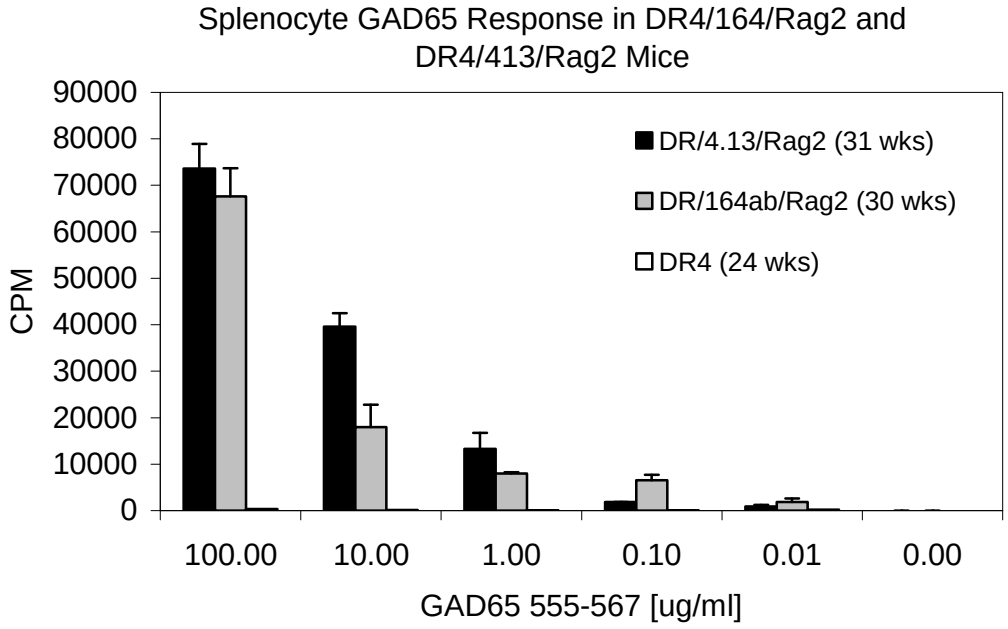

Figure 10. GAD65 555-567 dose response of 164 and 4.13 T cells from $Rag2^{o/o}$ mice. Thymidine was added at 72 hours and read at 96 hours.

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Autoreactive human T-cell receptor initiates insulitis and impaired glucose tolerance in HLA DR4 transgenic mice

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Abstract

A human T-cell receptor (TcR) derived from an autoreactive T-cell specific for GAD65, from a subject at high risk for autoimmune diabetes, was introduced into HLA-DR4 transgenic mice. The source of TcR was a $CD4^+$ T_H1^+ T-cell clone which responded to an immunodominant epitope of the human islet protein GAD65, an epitope shared with both GAD65 and GAD67 in the mouse. The resulting HLA-DR4/GAD-TcR transgenic mice on a $Rag2^{o/o}$/$I\text{-}Ab^{o/o}$/B6 background exhibited a $CD4^+$ infiltrate into pancreatic islets that correlated with a loss of insulin in infiltrated islets. These mice also exhibited a subclinical impaired tolerance to exogenously fed glucose as assayed by an intraperitoneal glucose tolerance test. T cells containing the GAD65/67 (555–567) responsive TcR undergo strong negative selection as evidenced by a 10-fold lower thymocyte cellularity compared to non-TcR transgenic mice, and clonotype peripheral T cells represented approximately 1% of $CD4^+$ T cells in Rag2 sufficient mice. Upon *in vitro* stimulation, GAD65/67 555–567 responsive T cells secrete interferon-γ, minimal interleukin (IL)-2 and tumor necrosis factor-α, and no IL-4, IL-5, IL-10, or IL-17, consistent with a T_H1 profile. These data demonstrate that $CD4^+$ T cells specific for a naturally processed epitope within GAD can specifically home to pancreatic islets and lead to impaired islet β-cell function in diabetes-associated HLA-DR4 transgenic mice on the relatively non-autoimmune C57BL/6 background. The relatively slow progression and patchy insulitis are reminiscent of the chronic pre-clinical phase similar to a majority of human at-risk subjects, and models these indolent features of human T1D.

Keywords: Insulitis; Human T-cell receptor; HLA transgenic mouse; GAD

1. Introduction

The human HLA-DQB1*0302 and -DRB1*04 gene products are strongly associated with autoimmune diabetes, and are also powerful susceptibility genes predisposing to diabetes when expressed as transgenes in the absence of endogenous class II ($I\text{-}Ab^{o/o}$) in the relatively non-autoimmune prone C57BL/6 mouse [1–3]. We recently described an age-dependent spontaneous loss of tolerance to an epitope within a naturally processed region of the diabetes autoantigen GAD65 (GAD65 555–567) in the presence of the autoimmune accelerator RIP-B7 in these diabetes prone DR4 transgenic mice. The loss of tolerance to GAD65 555–567 precedes overt hyperglycemia and is associated with a loss in glucose tolerance evidenced by an intraperitoneal glucose tolerance test [2,4].

Previous studies using immunization with putative human autoantigens (primarily GAD and insulin) in HLA transgenic mice have been used to identify, correlate, and confirm human T-cell reactive antigenic epitopes that may be targets for autoreactive T cells [5–8]. The spontaneous islet autoimmunity in the B7/DR4 C57BL/6 mouse, however, offers the opportunity

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to explore mechanisms of T-cell selection and autoreactivity in an unimmunized context. Glutamic acid decarboxylase exists in GAD65 and GAD67 isoforms, with GAD65 the predominant expressed form in human islets and GAD67 in murine islets [9]. It is important to note that GAD65 (555–567) is an ideal epitope for translational studies, as this epitope sequence is identical in all forms of mouse and human GAD (65 and 67). While cellular and humoral reactivity to glutamic acid decarboxylase 65 (GAD65) is readily detected in human T1D and diabetes-at-risk subjects [10–12], its direct role in the pathogenesis leading to islet insulin-producing β-cell destruction in the human disease is still uncertain. Antibodies to GAD65 are one of three serum antibody markers used in determining susceptibility to T1D in genetically predisposed individuals and imply a temporal relationship between immune reactivity to GAD65 and progression to human diabetes [13,14].

The common murine model for T1D, the NOD mouse, only partially recapitulates this pattern. GAD-mediated tolerogenic protection of diabetes in NOD mice can be afforded by intrathymic injection of GAD protein [15–17], inoculation with GAD65 encoding vaccinia virus [18], rat insulin promoter driven GAD65 [19], and antisense expression of GAD [20]. On the other hand MHC class I promoter-driven expression of GAD65 was shown to exacerbate disease [21]. In T-cell directed studies, a GAD65 responsive cell line has been shown to induce diabetes in NOD.*scid* mice [22] and recent evidence indicates that GAD epitopes are capable of stimulating diabetes-inducing BDC2.5 T cells and cause diabetes in transfer studies [23,24]. However, a protective role of cellular reactivity to GAD65 was shown to delay diabetes when interferon (IFN)-γ and interleukin (IL)-10 producing GAD65 responsive T cells were transferred in NOD mice from either T-cell transgenic mice [25,26], a T-cell clone [27] or a T-cell line [28]. Unaltered diabetes progression in NOD mice has also been observed in retrogenic expression of other I-Ag7-restricted GAD T-cell receptors [29].

In order to assess the characteristics of anti-islet T-cell specificities which are prevalent in human T1D we have transgenically expressed a GAD65 (555–567) responsive human T-cell receptor (TcR), derived from a diabetes at-risk individual, in DR4 transgenic mice on the C57BL/6 background. We report here that T cells in DR4 mice expressing TcR transgenes specific for GAD65 are strongly negatively selected in the thymus and are limited in numbers in the periphery organs. The percentage of $FoxP3^+$ cells among $CD4^+$ T cells in peripheral organs is about 3-fold greater than non-TcR transgenic mice. TcR transgenic mice were normoglycemic to 40 weeks of age, possibly related to the increase in selected regulatory components. Notably, mice transgenic for this TcR on a Rag2 deficient background are also normal for blood glucose but do exhibit insulitis at around 25 weeks of age that is correlated with a loss in glucose tolerance in an intraperitoneal glucose tolerance test and a loss in immunoreactive insulin in infiltrated islets. Thus, an anti-GAD T-cell specificity associated with human T1D is sufficient to elicit insulitis and impair glucose tolerance in a HLA-transgenic murine model, even in the context of the relatively autoimmune disease-resistant C57BL/6 background. In contrast to the autoimmune-prone NOD model, disease is indolent, insulitis is patchy, and lack of progression to overt diabetes is associated with evidence of T-cell regulation, features which may correspond to a large segment of the human T1D and at-risk population.

2. Research design and methods

2.1. Mice

DR0404-IE mice (DR4) were generated as previously described [2]. These C57BL/6 I-$Ab^{o/o}$ mice express a human-mouse chimeric class II molecule in which the TcR interacting and peptide binding domains of mouse I-E (domains α1 and β1, exon 2 in both genes) have been replaced with the α1 and β1 domains from DRA1*0101 and DRB1*0404 respectively. Retention of the murine α2 and β2 domains allows for the cognate murine CD4-murine MHC interaction [30].

The GAD65 (555–567) responsive human $CD4^+$Vα12.1/Vβ5.1 T-cell clone 164 was cloned from an HLA DR4 diabetes at-risk individual as previously described [12]. Human-mouse chimeric TcR transgenes were constructed by subcloning PCR amplified regions encoding rearranged VαJα and VβDβJβ domains from the human clone derived TcR sequences into pTαcass and pTβcass TcR transgenic vectors, respectively [31]. TcR transgenic vectors pTαcass and pTβcass contain the natural mouse TcR α and β promoter/enhancer elements and mouse Cα and Cβ constant region, respectively. DNA injection into C57BL/6/I-$Ab^{o/o}$ mouse embryos was performed at the University of Washington (Seattle, WA) in the Comparative Medicine animal facility. Founder mice containing the GAD65 TcR transgene were then crossed onto DR0404-IE mice to generate DR4/164 mice. Additional crosses were made onto Rag2 KO mice.

Blood glucose was performed via saphenous veins bleeds using a One-Touch FastTake glucometer (LifeScan, Milpitas, CA). All animal work was approved by the Benaroya Research Institute (BRI) Animal Care and Use Committee (ACUC) and animals were housed in the BRI AAALAC-accredited animal facility. For intraperitoneal glucose tolerance tests (IPGTT) mice were fasted (given water only) for 6 h. At the end of 6 h mice were injected intraperitoneal with 1.0 mg/ml D-glucose (stock solution in PBS) at a dose of 1 g/kg body weight. Saphenous blood glucose readings were taken at 0, 15, 30, and 60 min time points post injection.

2.2. Tissue processing and flow cytometry

Thymus, spleen, and lymph node tissues were processed into single cell suspensions by gently pressing through 0.40 μm cell strainers (BD-Falcon REF 352340, Bedford, MA) using the rubber end of a 1 ml tuberculin syringe in DMEM-10 media (DMEM cat. #11965-092; Gibco, Rockville MD) supplemented with 10% FBS (Hyclone, Logan, UT), 100 μg/ml penicillin, 100 U/ml streptomycin, 5 μM βme, 2 mM glutamine and 1 mM sodium pyruvate (Gibco, Rockville

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MD). Cell suspensions were centrifuged at 200 × *g* for 10 min and resuspended in DMEM-10 media. Splenic RBC were lysed using ACK lysis buffer [32] for 5 min at 37 °C at which time ~25 ml of media was added and cells spun down (200 × *g*). The following chromophore-labeled antibodies were used in flow cytometric analysis: anti-mouse CD4 (clone RM4-5), CD8 (clone 53-6.7), CD25 (clone PC61), CD62L (Mel-14), CD44 (IM7, BD-Pharmingen, San Jose, CA), anti-human Vβ5.1-PE (clone IMMU 157 Immunotech-Coulter, Miami, FL) and Vα12.1-FITC (clone 6D6; Endogen Woburn, MA). FACS samples were stained in media on ice for 45 min, washed once, and resuspended in FACS stain buffer (PBS containing 1% FBS, 0.1% Na-azide) before being run on a FACSCaliber or LSR II flow cytometer (Becton Dickinson). Internal staining of cells for FoxP3 was performed using eBioscience kit (FJK.16a Ab, San Diego, CA) according to the manufacturer's instructions.

Pancreatic tissues were in either: (1) fixed in phosphate-buffered formalin prior to paraffin embedding for H&E staining or insulin staining or (2) frozen in Tissue-Tek OCT embedding media (Sakura Finetek, Torrance, CA) for immunofluorescence. For immunofluorescence staining of frozen tissues, 6 μM tissue slices were fixed for 10 min in 4 °C acetone and either air dried and stored at −20 °C or stained directly. Frozen tissues section were blocked, stained, and washed in PBS containing 0.1%NaN_3/1%FBS/2% horse serum. The following antibodies were used at 1:100 dilution: CD4-Alexa-fluor 488 (MCD0420), CD8-Alexa-fluor 488 (MCD0820), control Alexa-fluor 488 (R2a20, Caltag, Burlingame, CA). Islet insulin was detected with primary guinea-pig polyclonal anti-insulin (1:100 dilution, Abcam Ab7842-500, Cambridge, MA) and a secondary goat anti-guinea-pig Alexa-fluor 568 (1:100 dilution, Molecular Probes, Eugene, OR). Immunofluorescence was detected on a Leica DM IRB microscope. For islet infiltrate scoring, at least 8 islets were viewed for each mouse and H&E stained islets we scored as follows: 0, no infiltrate; 1, less than 33% infiltrated; 2, less than 66% infiltrated; and 3, greater than 66% infiltrated.

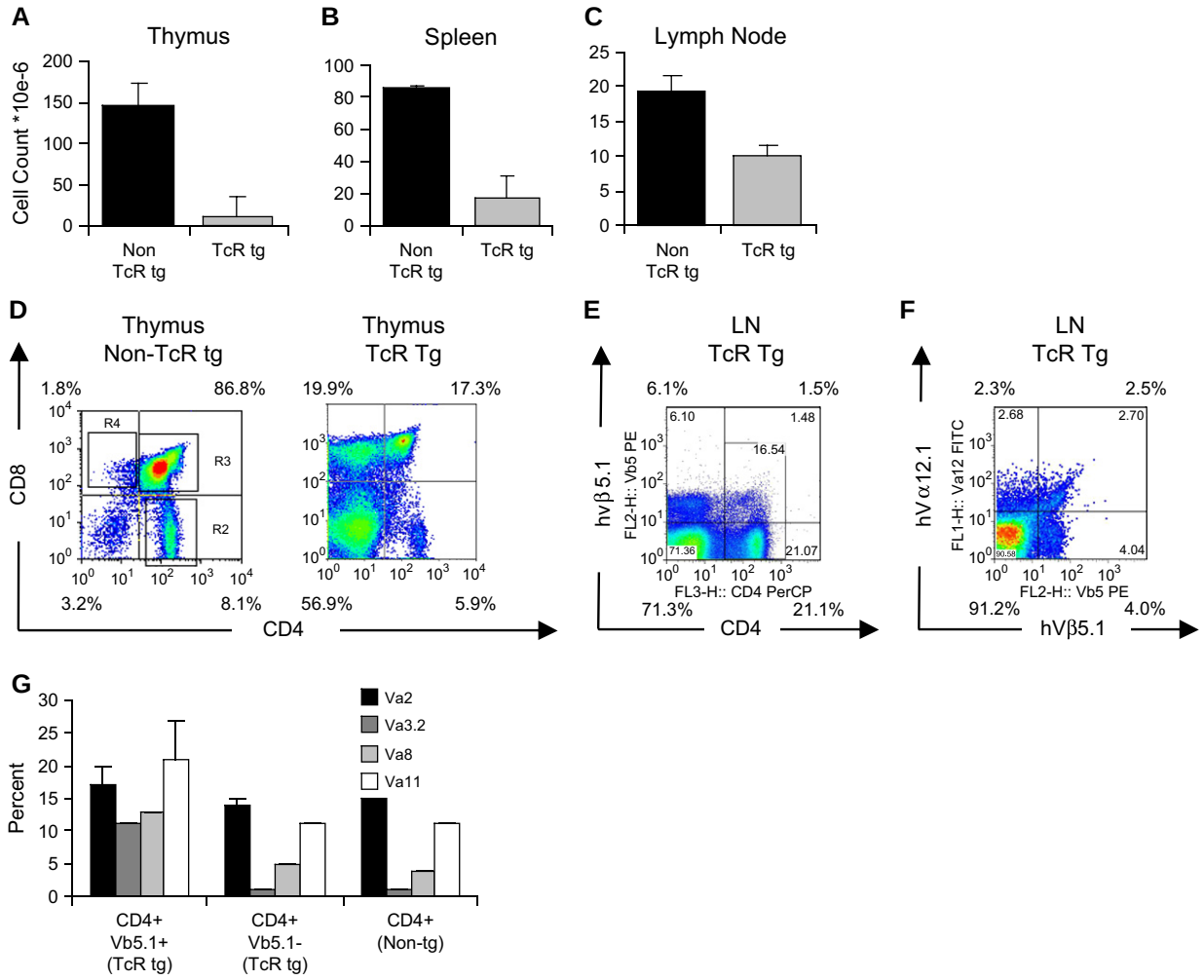

Fig. 1. Tissue cellularity and GAD TcR expression in DR4/164 GAD65 555–567 specific TcR transgenic mice. Cellularity for Thymus (A), Spleen (B), and Lymph nodes (C) represent the average counts ($n = 3$ mice each) for Non TcR transgenic (Non TcR tg) and TcR transgenic mice (TcR tg) at 8 weeks of age. CD4 vs. CD8 profiles in thymus of DR4 and DR4/164 GAD TcR mice (D). Expression of human TcR Vβ5.1 transgene on $CD4^+$ lymph node cells from the DR4/164 mouse is shown (E) along with clonotypic expression of the Vα12.1/Vβ5.1 TcR on CD4-gated lymph node cells (F). $CD4^+$ human $V\beta5.1^+$ transgenic T cells express an increase in the use of endogenous mouse TcR Vα compared to $V\beta5.1^-$ T cells (G).

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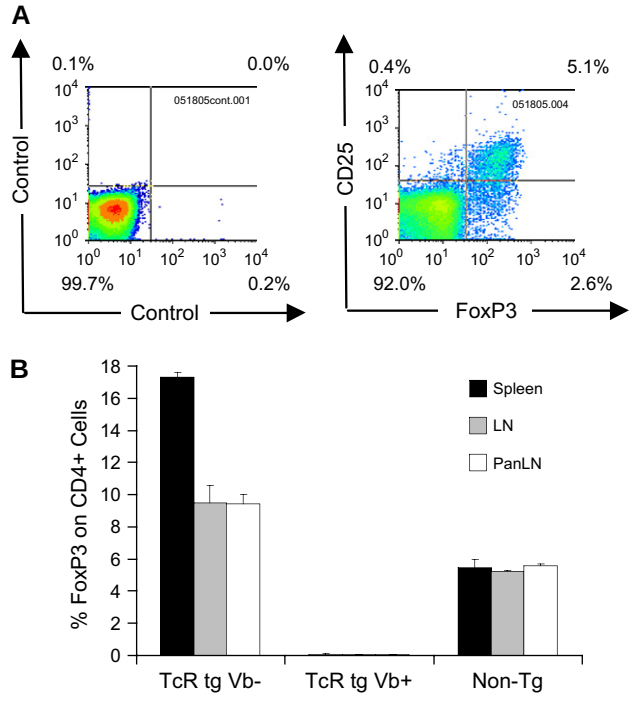

Fig. 2. Intracellular FoxP3 expression on $CD4^+$ T cells in GAD65 555–567 TcR transgenic and non-transgenic DR4 mice. FoxP3 expression in lymph node cells from DR4/164 TcR transgenic mice showing a correlation of FoxP3 expression with CD25 expression on $CD4^+$ gated cells (A). Average percentages from 3 mice (8 weeks of age) of $CD4^+$ $FoxP3^+$ expressing cells of all $CD4^+$ cells (B). Percentages are shown for $CD4^+/V\beta5.1^+/FoxP3^+$ (Tg $V\beta^+$) and $CD4^+/V\beta5.1^-/FoxP3^+$ ($TgV\beta^-$) for DR4/164 GAD-TcR mice and for non-TcR transgenic mice (Non-tg).

2.3. Proliferation assays

Single cell suspensions of lymph node cells (LNC) from inguinal, mesenteric and brachial lymph nodes and spleen cells were prepared by gently pressing through 0.4 μm nylon cell strainers (BD-Falcon REF. 352340, Bedford, MA) in Hanks buffer (Gibco, Rockville, MD) and spun down (1000 rpm, 200 × *g*). Splenic RBC were lysed using ACK lysis buffer [32] for 5 min at 37 °C at which time ~25 ml of media was added and cells spun down (200 × *g*). Splenocytes were resuspended in DMEM-10 (DMEM cat. #11965-092; Gibco) supplemented with 10% FBS (Hyclone), 10 μg/ml penicillin, 100 U/ml streptomycin, 50 μM βme, 2 mM glutamine and 1 mM sodium pyruvate (Gibco). In lymph node proliferation assays 1e5 lymph node cells were cultured with 2e5 3000 Rad Cs-g irradiated splenocytes. Supernatants for cytokine analysis were taken (50 μl) at 48 h and μCi/well of [^{3}H]thymidine was added at 72 h. Thymidine incorporation was assayed at 96 h using a liquid scintillation counter analyzed on a scintillation counter (Wallac–Perkin/Elmer Life Sciences, Boston, MA) at 96 h. Splenocyte responses were measured in the same manner using 5×10^5 splenocytes per well.

2.4. Cytokine analysis

Cytokines IL-2, IL-4, IL-5, tumor necrosis factor (TNF)-α, and IFN-γ were assayed using a Mouse Th1/Th2 Cytokine CBA kit (BD Bioscience, San Diego, CA, cat. #551287). IL-10 was assayed using a BD OptEIA mouse IL-10 Elisa Set (BD Bioscience, cat. #555252) and IL-17A was assayed using an IL-17A ELISA kit (eBioscience, San Diego, CA, cat. #88-7147-22). Supernatants from triplicate proliferation wells (50 μl/well) were combined for cytokine analysis with 50 μl used for CBA analysis and 50 μl each for IL-10 and IL-17A ELISA.

3. Results

GAD65 (555–567) is a minimal stimulating epitope within a naturally processed immunodominant epitope (GAD65 552–572) within the diabetes autoantigen GAD65 [7,33]. In studying T1D in human diabetes-correlated HLA transgenic mice, the MHC DR4-binding GAD65 (555–567) epitope is an autologous antigen as mouse and human sequences in GAD65 and GAD67 are all identical [34]. A human $CD4^+V\alpha12.1/V\beta5.1$ TcR (Arden nomenclature [35]) T-cell clone (164) responsive to GAD65 (555–567) was derived from PBMC of an autoantibody-positive diabetes at-risk individual by in vitro stimulation with a GAD65 (555–567) superagonist APL peptide and single cell sorted from a $CD4^{Hi}/CD25^+$ activated population [12]. 164 TcR transgenic mice were generated using

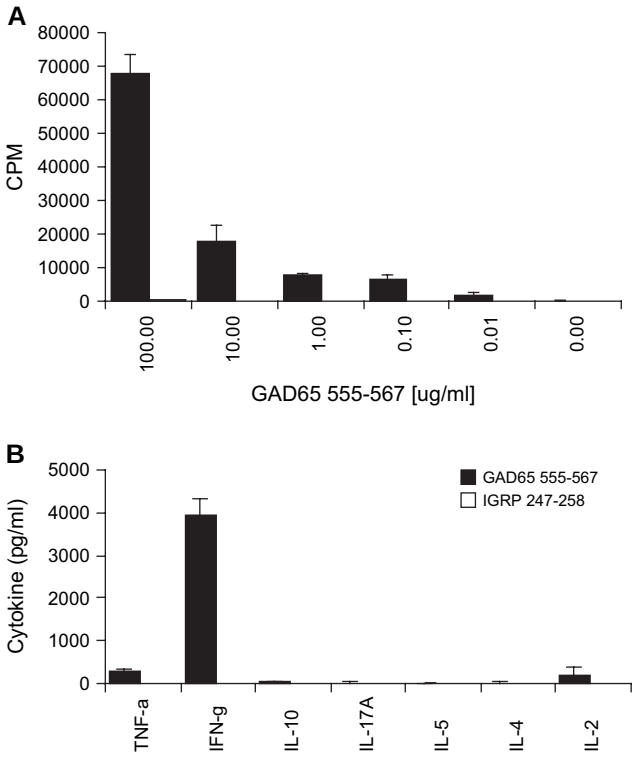

Fig. 3. Antigen-specific dose–response and cytokine analysis of in vitro stimulated 164 GAD TcR T cells. Splenocytes from Dw14/164/$Rag2^{o/o}$ mice (2e5) were stimulated with increasing amount of GAD65 555–567 or control IGRP 247-258 peptide for 96 h. [^{3}H]Thymidine was added at 72 h and incorporation was assayed by scintillation counting (A). Cytokines (pg/ml) from supernatants from proliferation assays (at 100 μg/ml antigen) taken at 72 h (B). The experiment was repeated 3 times with similar results.

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murine TcR cassettes pTαcass and pTβcass [31] in which the variable regions of the mouse TcR were substituted with the human sequences from the 164 human clone TcR. Purified DNA was microinjected directly into C57BL/6 embryos. TcR positive founder mice were crossed onto (I-Ab$^{o/o}$) C57BL/6 DR4 HLA transgenic mice.

3.1. Thymic and peripheral cellularity in GAD65 555–567 responsive TcR transgenic mice

Thymocyte cellularity in DR4/164 TcR transgenic mice is severely reduced compared with non-TcR mice, indicating a threshold in negative selection has been crossed in selection of the 164 TcR. (Fig. 1A). In wild type DR4 mice the CD4:CD8 single positive ratio in the thymus is approximately 4:1, the CD4:CD8 ratio in the TcR negative selecting DR4/164 mouse is 1:3.4 and the percentage of CD8 single positive cells in the thymus is ~20% or nearly 10-fold above that seen in wild type DR4 mice (Fig. 1D). A similar type of CD8 skewing has been observed on other self antigen specific TcR transgenic mice [36–38]. The presence of the GAD65 (555–567) specific TcR transgenes in DR4 mice results in an increase in the thymic CD4$^-$/CD8$^-$ population from less than 5% in wild-type DR4 mice to nearly 60% in DR4/164 mice (Fig. 1D).

A result of the extensive negative selection in the thymus of DR4/164 mice is reflected in the peripheral organs where both splenic and lymph node cellularity are well below non-TcR DR4 mouse levels (Fig. 1B and C). In the peripheral lymph nodes the expression of the Vβ5.1 transgene is found on about 6% of CD4$^+$ T cells (Fig. 1E) and these CD4$^+$/Vβ5.1$^+$ T cells display an increase usage of endogenous Vα TcR compared to CD4$^+$/Vβ5.1$^-$ cells (Fig. 1G). The percentage of peripheral CD4$^+$ T cells expressing the clonotypic Vα12.1/Vβ5.1 transgene is around 2% (Fig. 1F).

3.2. 164 TcR transgenic mice have an increased percentage of FoxP3 positive T regulatory cells

Several other models using TcR transgenic mice have shown an increase in the percentage of FoxP3 expressing cells in the population of T cells responding to antigens which are introduced by transgenesis, and are therefore surrogates for self antigens [39,40]. Consistent with these observations, the percentage of FoxP3 positive cells in the CD4$^+$CD25$^+$ subset of DR4/164 mice is greater in DR4/164 mice compared to non-TcR transgenic DR4 mice (Fig. 2B). However, while an increase in the percentage of CD4$^+$/FoxP3$^+$ cells among the CD4$^+$ subset is observed in inguinal and pancreatic lymph nodes and also in spleen, the increase is only observed in the non-TcR transgenic Vβ5.1$^-$ population and not the Vb5.1$^+$/CD4$^+$ cells.

3.3. 164 TcR T cells are GAD65 responsive and exhibit a T_H1 phenotype

Proliferation and cytokine production of T cells from DR4/164/Rag2$^{o/o}$ mice in response to GAD65 (555–567) and a control peptide derived from diabetes autoantigen islet-specific glucose-6-phosphatase subunit related protein (IGRP 247–258) are shown in Fig. 3. Antigen specific response to GAD65 (555–567) is seen as low as 0.01 μg/ml (6.8 nM, lowest concentration tested) but not to control DR4-binding IGRP (247–258) peptide. A cytokine analysis in response to GAD65 (555–567) stimulation showed that these cells secrete IFN-γ with minimal TNF-α and IL-2 and no IL10, IL-17, IL-4, or IL-5, and is indicative of a T_H1 type cell cytokine profile.

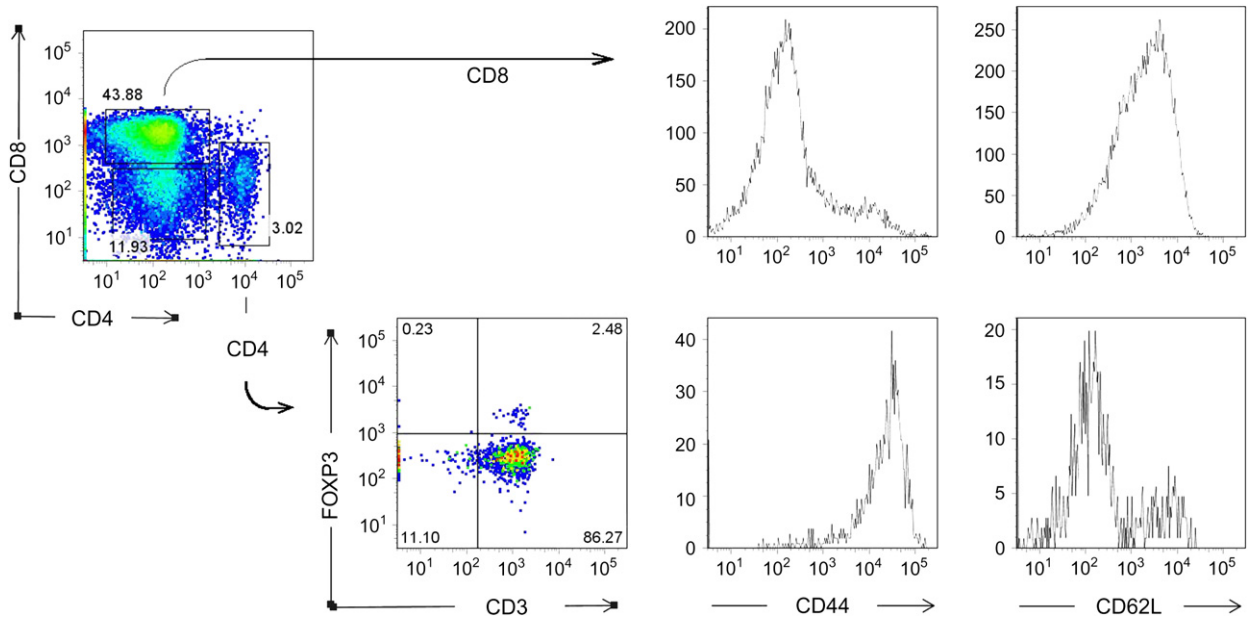

Fig. 4. TcR transgenic CD4$^+$ cells in DR4/164/Rag2$^{o/o}$ mice exhibit an activated phenotype. CD4$^+$ lymph node T cells, but not CD8$^+$ cells from DR4/164/Rag2$^{o/o}$ mice (10 weeks of age) are CD44^{Hi} and CD62L$^-$.

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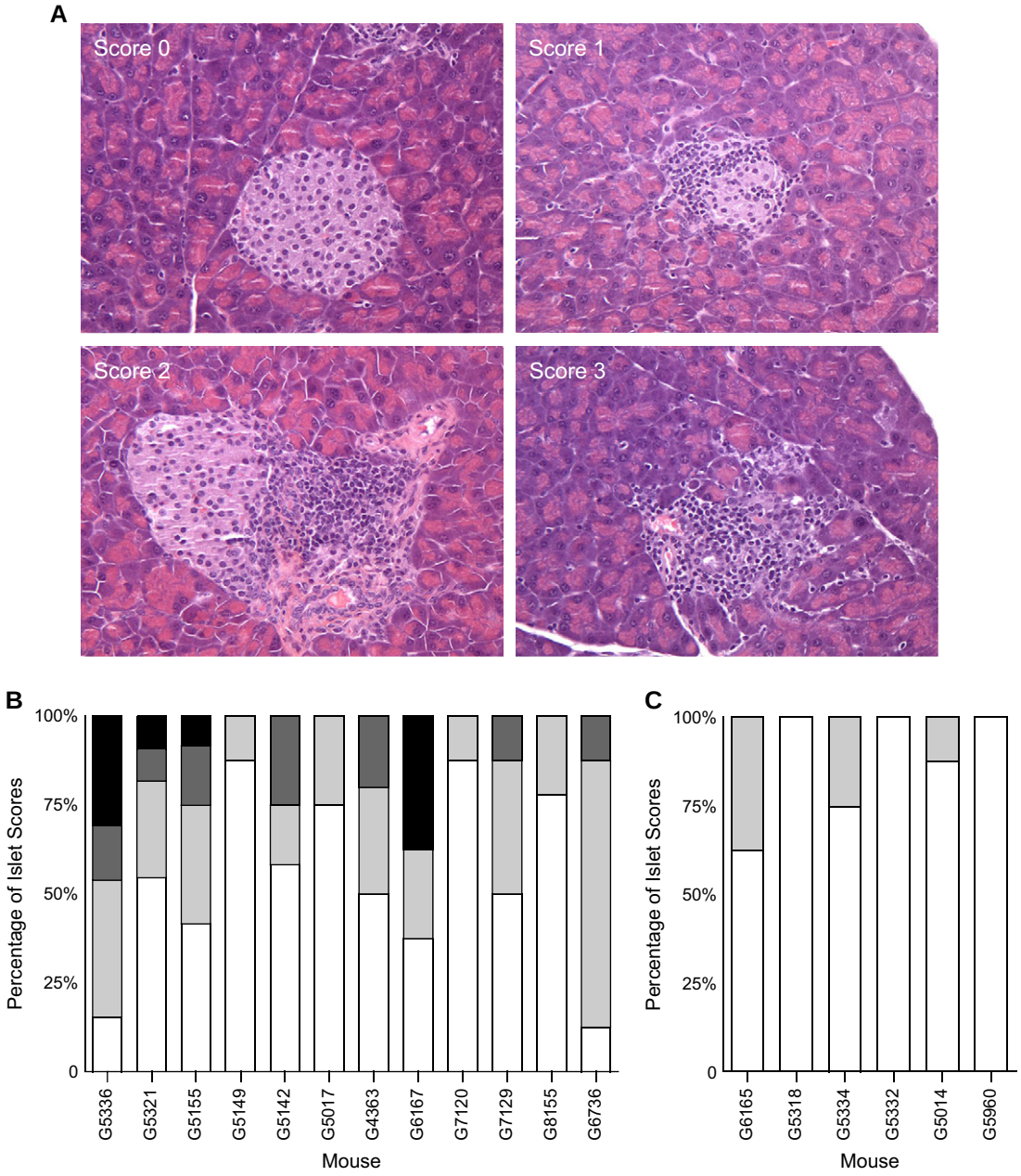

Fig. 5. Islet cell infiltrate in DR4/164/Rag2$^{o/o}$ TcR transgenic mice. H&E staining on formalin fixed paraffin embedded pancreatic tissue from a 28-week-old female DR4/164/Rag2$^{o/o}$ mouse (A). Islets were scored as 0, no infiltrate; 1, less than 33%; 2, less than 66%; 3, greater than 66%. A summary of islet infiltrate scores for 12 female and 6 male DR4/164/Rag2$^{o/o}$ mice is shown in (B) and (C) respectively. Islets were scored as 0, no infiltrate (white bars); 1, less than 33% (light gray bars); 2, less than 66% (dark gray bars); 3, greater than 66% (black bars).

The same cytokine profile is seen in Rag2 sufficient mice (data not shown) but proliferative values and detectable cytokines are lower, likely due to the large percentage of non-clonotypic T cells and FOXP3^{+} cells selected in Rag2 sufficient mice (Figs. 1E and 2B).

3.4. GAD65 555–567 TcR transgenic mice on a Rag2$^{o/o}$ background have insulitis

Blood glucose levels in DR4/164 Rag2 sufficient mice ($n = 20$) were monitored up to 40 weeks of age, and no mice showed overt hyperglycemia. Histological examination of pancreata from DR4/164 Rag2 sufficient mice also appeared normal with no indication of an islet infiltrate (data not shown). To increase the expression of the clonotypic transgenic GAD TcR, DR4/164 mice were crossed onto Rag2 knockout mice to generate DR4/164/Rag2$^{o/o}$ mice. As previously seen for Rag2$^{+/+}$ animals (Fig. 1D), concomitant with the strong negative selection of the 164 TcR in these mice on the Rag2$^{o/o}$ background, a peripheral skewing of the clonotypic TcR towards the CD8^{+} single positive T-cell lineage was observed (Fig. 4). However in contrast to the CD8^{+} T cells, the CD3^{+}/CD4^{+} T cells from DR4/164/Rag2$^{o/o}$ mice display an activated CD44^{Hi}/CD62L^{-}

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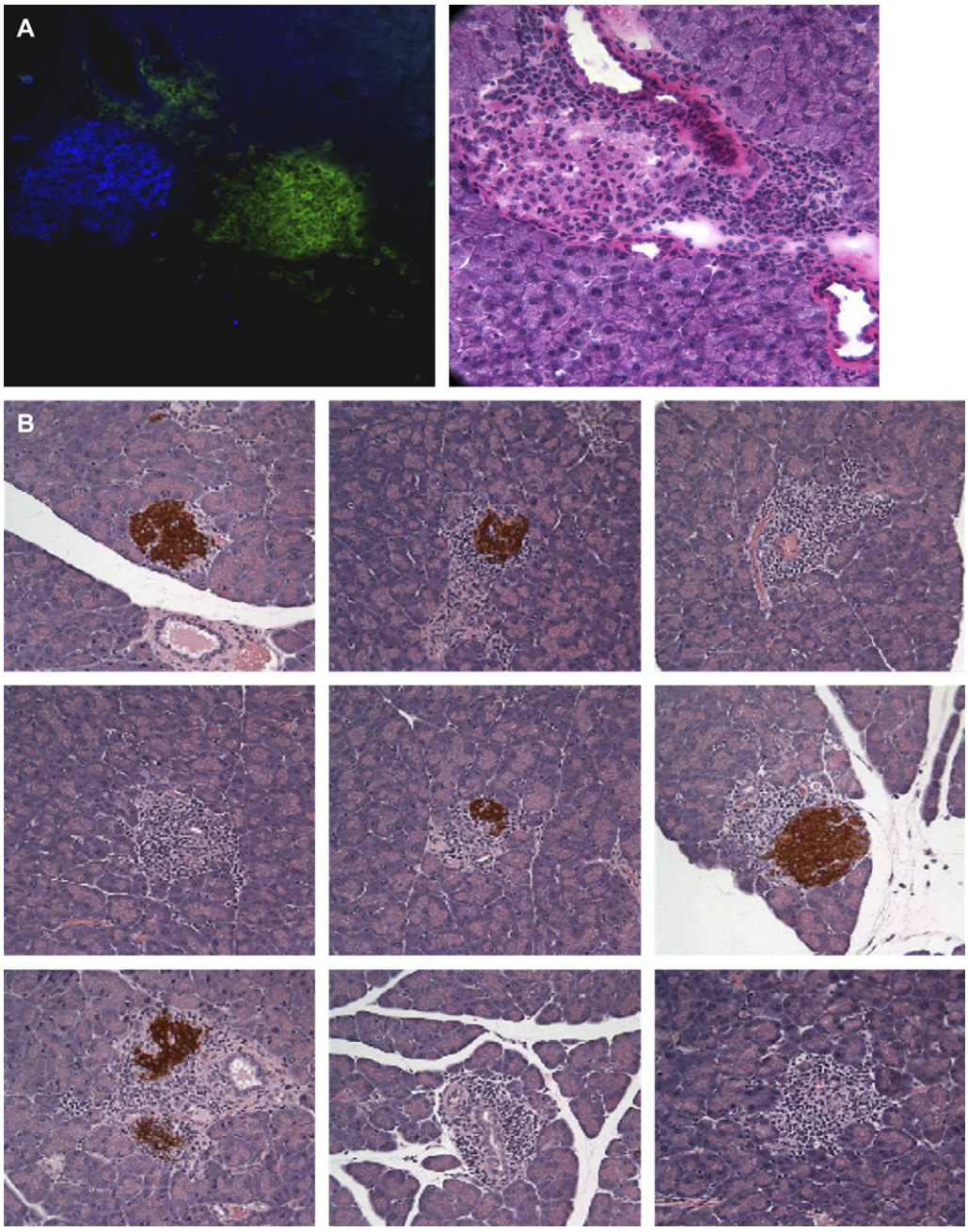

Fig. 6. Detection of $CD4^+$ cells and loss of insulin staining in infiltrated islets from DR4/164/$Rag2^{o/o}$ mice. Immunofluorescence staining for insulin (blue) and CD4 (green) in an infiltrated islet from a 28-week-old female DR4/164/$Rag2^{o/o}$ mouse (A). Immunohistochemistry staining for insulin (brown) in infiltrated islets from a female DR4/164/$Rag2^{o/o}$ mouse (B).

phenotype (Fig. 4). In addition, unlike the lack of FoxP3 expression on $V\beta5.1^+/CD4^+$ T cells in DR4/164/$Rag2^{+/+}$ mice (Fig. 2B), a fraction of peripheral $CD4^+$ cells in DR4/164 $Rag2^{o/o}$ do express FoxP3 (2.5%).

Unlike DR4/164/$Rag2^{+/+}$ mice, DR4/164/$Rag2^{o/o}$ mice exhibit an islet-specific cellular infiltrate into the pancreas beginning at about 25 weeks of age (Fig. 5A). The islet-specific infiltrate is observed primarily in female mice (Fig. 5B and C) and is not seen in other organs (supplemental data S1). Immunofluorescence staining of the islets showed $CD4^+$ staining indicative of the 164 T cells infiltrating into these islets (Fig. 6A). $CD8^+$ cells were not detected in the infiltrated islets (data not shown). Correlating with the cellular infiltrate in DR4/164/$Rag2^{o/o}$ islets is a loss of detectable insulin staining in most, but not all, islets (Fig. 6B). To assay if the loss of insulin staining in T-cell infiltrated islets is reflected in pancreatic function we performed an intra-peritoneal glucose tolerance test (IPGTT) on DR4/164/$Rag2^{o/o}$ mice. As shown in Fig. 7, DR4/164/$Rag2^{o/o}$ mice are impaired in their response to injected glucose at a time when they display a patchy T-cell infiltrate into the islets with loss of immunoreactive insulin in these islets.

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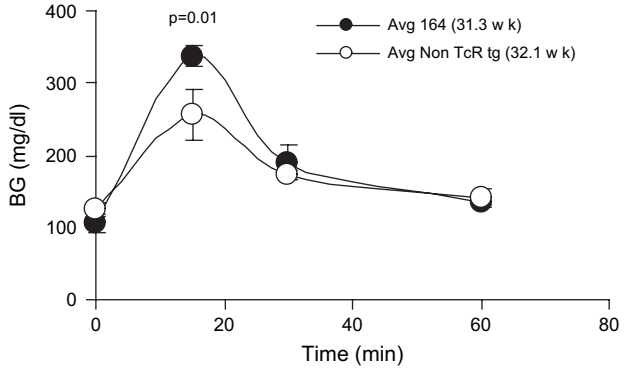

Fig. 7. Impaired response to exogenous glucose in DR4/164/Rag2$^{o/o}$ mice. DR4/164/Rag2$^{o/o}$ (164, filled circles) and DR4/Rag2$^{o/o}$ (Non TcR tg, open circles) mice were fasted for 6 h prior to glucose injection and blood glucose monitoring (see Section 2). Four mice 30 weeks of age were used in each group.

4. Discussion

HLA-DR4 MHC transgenic mice on a relatively non-autoimmune prone C57BL/6 background were evaluated for propensity to autoimmune diabetes by introduction of self-antigen specific GAD65 (555–567) responsive TcR transgenes. The TcR sequence originated from a human T-cell clone (164) derived from a diabetes at-risk individual and was chosen because: (1) the GAD65 (555–567) sequence is identical to both GAD65 and GAD67 forms in both mice and humans with the later form of GAD being the most dominant in the murine pancreas [9], and (2) the minimal stimulating GAD65 (555–567) sequence is within a naturally processed epitope in both mouse and man [7,33]. These 164 transgenic T cells in DR4 mice were strongly negatively selected against and weakly populate the secondary lymphoid organs. Nevertheless, peripheral transgenic T cells are antigen-specific for GAD65 (555–567) and display a T_H1 phenotype by expressing IFN-γ with minimal TNF-α and IL-2 and no IL10, IL-17, IL-4, or IL-5 upon in vitro challenge.

While GAD65 (555–567) TcR transgenic mice on HLA DR4 Rag2 sufficient background did not show evidence for loss in pancreatic function, GAD65 TcR transgenic mice on a Rag2$^{o/o}$ background (DR4/164/Rag2$^{o/o}$) beginning at about 25 weeks of age showed signs of impaired islet function, as demonstrated by a CD4$^+$ T-cell islet-specific infiltrate into the pancreas that was correlated with a loss in islet insulin staining and an abnormal response to an intraperitoneal glucose tolerance test. Thus, the GAD65 (555–567) specificity is sufficient for initiation of insulitis, resulting in metabolic compromise characteristic of pre-diabetes. Since this phenotype occurs on the relatively autoimmune resistant C57BL/6 background, it suggests that T-cell autoreactivity to GAD65 is sufficient for early immune activation associated with early autoimmunity to pancreatic islets, but that additional autoimmunity predisposition is likely to be necessary for full disease penetrance. This phenotype is distinct from that described by Tarbell et al. [26] and Kim et al. [25], in which GAD65 reactive I-Ag7-restricted TcR transgenic NOD mice appeared to the protected from diabetes and correlated with IL-10 and IFN-γ being secreted by CD4$^+$ T cells. A determination of whether the protection in those GAD TcR models was mediated by T-cell produced IL-10 was not addressed. Other islet responsive (including GAD) IL-10 producing T cells have been shown to protect from diabetes in transfer models [27,28,41] with the later study showing abrogating effects by blocking IL-10 signaling. In contrast, the insulitic behavior of 164 GAD T cells in DR4 mice shown here do not produce IL-10.

The FOXP3 expression profiles differed in DR4/164 TcR Rag2 sufficient and Rag2$^{-/-}$ mice. In the Rag2-sufficient animals, a substantial population of transgene-negative FOXP3$^+$ T cells was present in the periphery, and in spleen approximately 3 times the frequency found in the non-TcR transgenic DR4 mice. It is possible that these cells arise as a compensatory mechanism suppressing the subpopulation of transgene-positive cells; in any event, the presence of these FOXP3$^+$ cells correlated with a lack of insulitis or hyperglycemia. In the Rag2 deficient mice, we were therefore surprised to find a small percentage of clonotypic CD4$^+$/FoxP3$^+$ cells (Fig. 4). Studies are underway to evaluate the functional suppressive capacity of this small population in animals with insulitis, but lacking overt diabetes.

The insulitis phenotype of GAD TcR and HLA transgenic mice supports the hypothesis that T-cell reactivity to GAD restricted by a diabetes-associated human MHC molecule plays a role in diabetes or pre-diabetes pathogenesis. These mice had impaired glucose tolerance but did not become hyperglycemic; potential reasons could be that other T-cell specificities (or B cells) are required for disease progression, or alternatively that regulatory mechanisms in the context of the C56Bl/6 genome are sufficient to reduce penetrance. A requirement for B cells in non-TcR transgenic NOD diabetes has been established [42,43]. Mice are presently being crossed onto TcR C$\alpha^{o/o}$ mice to determine if the presence of B cells in the presence of clonotypic 164 T cells will lead to hyperglycemia. The results from this initial study indicate that T_H1 GAD specific T cells can spontaneously migrate specifically to pancreatic islets in DR4 humanized mice on a relatively non-autoimmune background and are capable of mediating a loss in β-cell function.

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Appendix I. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jaut.2007.08.001.

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